

Identification of sentinel plant species for evaluating phytotoxicity of veterinary antibiotics in mediterranean europe

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Abstract

Background

Antibiotics used to treat livestock species enter agricultural fields when they are excreted by grazing animals or are present in manure that is added to fields as fertiliser. In the European Union, the potential effects of such antibiotics on terrestrial plants must be evaluated following the standardised OECD 208 method, which specifies the crop and wild species that should serve as “sentinels” for assessing antibiotic exposure. The present study aimed to compare this approved list of sentinel species against crop and wild plant species actually present in agricultural and pasture lands in Mediterranean Europe in order to identify the most appropriate sentinel plants for the region. The study focused on Spain as a region representative of Mediterranean Europe. Georeferenced layers for wild plant species and cultivated areas (crops), livestock density and land cover were combined, and then selection criteria were applied, leading to the identification of sentinel crop species for agriculture and pasture scenarios.

Results

In the agriculture scenario, the sentinel crop species were barley, wheat, corn, sunflower, dried pie, alfalfa, vetch, oilseed rape and sugar beet; the sentinel wild species were *Papaver rhoeas*, *Galium aparine* and *Chenopodium album*. In the pasture scenario, sentinel wild species were *Bromus tectorum*, *Agrostis capillaris*, *Trifolium pratense*, *Lotus corniculatus* and *Galium aparine*. The following common weed species in field boundaries or in pasture lands also emerged as potential sentinel species for risk assessment, even though they are not listed in the OECD 208 method: *Sonchus oleraceus*, *Avena sterilis*, *Dactylis glomerata*, *Hordeum murinum* and *Lolium rigidum*.

Conclusions

The sentinel species identified in this study may be useful in risk assessment procedures covering Mediterranean Europe. The method developed for this study could be useful for identifying sentinel species for other geographical areas.

1. Background

Plant biodiversity plays a critical role in biogeochemical cycles and provides important ecosystem services [1, 2]. It is essential for capturing nutrients and retaining soil, and it provides habitats for animal species [3, 4], including pollination species and predators of pest species, serving as biological control against insects and weeds [5–8]. Biodiversity also provides humans with food, helps purify water supplies, generates oxygen and supplies raw materials for building, clothing, paper and other products [9].

There are around 500,000 species of land plants in the world [10], nearly 20% of which have been at risk of extinction since 1900 [1, 11]. The drastic decrease in species diversity in Europe during the 20th century is closely linked to anthropogenic activities [12] that have led to habitat loss, fragmentation, degradation and overexploitation, species invasion, pollution, climate change and reduced availability of water [2, 13].

In fact, the largest contributor to biodiversity loss may be agriculture [4, 14, 15], which exerts direct pressures on biodiversity [16]. Agricultural land area has increased about 300% since 1970, such that it now occupies a third of the world's surface land [17]. Indeed, 37% of the total land within the European Union (EU) is used for agriculture, with 12% of the total land serving as crop land to grow plants for human and animal consumption, while 25% serves as pasture land for grazing animals [4]. The area devoted to agriculture has increased in parallel with the cultivation of selected plant species and varieties in extensive, homogeneous landscapes. For example, a shift in many EU countries from semi-natural to cultivated grasslands that serve as pastures and meadows for livestock grazing or animal feed production has reduced plant biodiversity [18]. This biodiversity loss has resulted from grazing pressure, fertilisation, cutting management and the increasing dominance of competitive plant species [12, 18–20]. At the same time, mechanised agricultural practices and use of fertilisers and pesticides have increased. All these factors have eroded the quantity and quality of habitat for many plants and animals, reducing their abundance and biodiversity [21–23].

Agricultural land is at high risk of contamination by antibiotics used to treat livestock species, and the antibiotics can harm crop plants [24–31] and wild plants [27, 28, 31, 32]. Antibiotics are poorly absorbed by animals, so 30-90% is excreted in manure that is added to the field immediately by grazing animals or later when it is added to fields as fertiliser [33–35]. Antibiotics pass from the manure into the soil. Studies often using higher antibiotic concentrations than those expected in agricultural soil [36, 37] have suggested that the compounds can harm plants mainly by delaying or inhibiting germination; inhibiting enzyme activity; stunting growth of shoots, roots, and stems; reducing the number of leaves and biomass production; and inducing necrosis and death [37, 38]. Minden et al. [38] observed delayed germination of crop and wild species after exposure to antibiotic concentrations typically found in agricultural and grassland soil. Ultimately, antibiotics may reduce plant species richness, abundance and/or diversity in treated fields as well as adjacent habitats, such as field margins and grasslands [38], with secondary effects on species at higher trophic levels [39].

The EU mandates assessment of the risk that antibiotics used to treat livestock may exert environmental effects (Directive 92/18/EC). This assessment should be carried out according to the standardised OECD 208 method, based on sentinel terrestrial plant species drawn from an approved list of 32 crop species or 52 wild species. Current recommendations are to include four or five dicotyledonous (dicot) and two or three monocotyledonous (monocot) crop species in the risk assessment [41], in order to adequately represent the types of plants grown on agricultural land fertilised with manure. However, we [42] and others (Küster and Adler, 2014) have argued for inclusion of the Poaceae family as the most representative crop family, given that cereals for human and animal consumption are grown on 50% of

EU agricultural land [44]. We have also argued for including wild species in risk assessments in order to ensure the broadest protection of plant biodiversity [42].

In the present study, we drew on geographical information system mapping data to catalogue all crop species and wild plant species listed in the OECD 208 method on agricultural and pasture lands in a representative area of Mediterranean Europe. Then we applied selection criteria to the catalogue in order to identify the species potentially most suitable as sentinels for inclusion in risk assessments of environmental effects from antibiotics used to treat livestock. We selected Spain as a representative country in Mediterranean Europe. Most (85.5%) of the country is found in the Mediterranean biome, and the remaining 14.5% belongs to the temperate biome [45], which covers the north of the Iberian Peninsula [46]. Spain is home to areas of high terrestrial biodiversity [47] as well as hotspots of valuable agricultural and pasture landscapes [48]. The country ranks second in the EU in terms of harvested area (around 25,000,000 ha) and agricultural production (25 billion EUR) [49], and it contains the largest proportion (33%) of all permanent grasslands in the EU [50, 51]. Spain contains the second largest livestock population in the EU [44], and the animals are managed under a range of systems. The country ranks first in the EU for the number of pigs and third for the number of bovine animals, and it is the second-largest producer of pigs (4.6 million tonnes) and poultry (1.7 million tonnes) [49].

2. Methods

Major crop and wild plant species on agricultural and pasture lands where antibiotics are used to treat livestock were identified in two steps. First, agriculture and pasture scenarios receiving manure from intensively reared or grazing animals were identified and mapped. Second, data on crop species and wild plant species present in those scenarios were analysed to determine richness, diversity and evenness in the case of wild species, or number of cultivated hectares in the case of crop species. These steps are detailed in the subsequent paragraphs. All analyses were conducted using ArcMap 10.7 [52] and R 3.6.3 [53].

- **Definition of scenarios**

We considered the agriculture and pasture scenarios described by the “Guideline on Environmental Impact Assessment for Veterinary Medicinal Products” [54]. Agriculture scenarios were defined as crop fields that lie adjacent to intensive animal facilities and that receive manure from the intensively reared animals. Pasture scenarios were defined as lands covered with grass or herbage and grazed by livestock. In the agriculture scenario, we considered crops and wild species present in adjacent habitats of crop fields, such as field margins; in the pasture scenario, we considered only wild species because grazing animals consume forage, which is incompatible with crop production.

- **Mapping of agriculture and pasture scenarios**

The two scenarios were mapped by combining georeferenced layers on livestock density and land cover. To build density layers of intensively reared or grazing animals, georeferenced point data on cattle, pigs,

and chickens were obtained from the Spanish Register of Livestock Holdings (REGA database, General Directorate of Livestock, Spanish Ministry of Agriculture, Fisheries and Food). This database includes data on whether the animals are raised indoors or outdoors. We assumed that “indoors” equated to “intensively reared”, while “outside” equated to “grazing”, except in the case of chickens, all of which we classified as intensively reared because chickens are typically grown in fenced yards and manure surplus is applied to agricultural fields [55]. In this way, we built five kernel density layers: intensively reared cattle, grazing cattle, intensively reared pigs, grazing pigs and intensively reared chickens.

We extracted population values from the livestock census, and we applied Silverman’s Rule of Thumb [56] to define the search radius of each kernel density layer (Table A1):

$$Search\ radius = 0.79 * \min \left(SD, \sqrt{\frac{1}{\ln(2)} * D_m} * n^{-2} \right)$$

where SD is the standard distance; D_m , the median of the distances; and n , the total census.

We assumed that the probability that manure was applied to a given area was inversely proportional to the distance from an intensive animal facility, whose locations we extracted from the REGA database [57]. This reflects that farmers typically spread manure on their own land or on nearby land to avoid the costs and environmental disturbance (mainly outdoor smells) of transporting it farther away [58–60]. Similarly, we considered that the probability of grazing was inversely proportional to the distance from a primary animal location. In this way, each kernel animal density layer was categorised as having a low, medium or high manure load, with the three categories defined based on tertiles after exclusion of 0 values.

Layers of intensive crop and grassland cover were obtained from the Spanish Land Cover / Land Use Information System (SIOSE in Spanish; CNIG, 2014), after reclassifying the Inspire categories (CODIIGE). SIOSE is a polygon-based spatial dataset of land cover in the country at a scale of 1:25,000, and it is the basis for Spanish CORINE land cover data [62]. The dataset offers a minimum mapping unit of 2 ha for agricultural land, forests and natural areas. Land covers corresponding to crops were selected according to Piñeiro et al. [63] and the Spanish Ministry of Agriculture Food Security and Environment [64]. Land covers corresponding to grasslands were selected according to Ferrer et al. (2001; Table A2).

The livestock density layers were combined with the land cover layers using the mask tool in ArcGIS [52] in order to generate the agriculture and pasture scenarios. During this process, intensively reared animal layers were combined with the crop layer, whereas grazing animal layers were combined with the grassland layer. We constructed separate maps of the agriculture scenario for pigs, cattle and chickens; as well as separate maps of the pasture scenario for pigs and cattle. Each map classified areas by manure load. Altogether we considered 15 “subscenarios” defined by scenario (agriculture or pasture), animal species (pigs, cattle, chickens) and manure load category (low, medium, high) (Table 1).

Table 1. Subscenarios in the area of study, defined by scenario, animal species and manure load

Scenario	Animal species	Manure load	Subscenario
Agriculture	Pig	High	Agri_Pig_H
		Medium	Agri_Pig_M
		Low	Agri_Pig_L
	Cattle	High	Agri_Cattle_H
		Medium	Agri_Cattle_M
		Low	Agri_Cattle_L
	Chicken	High	Agri_Chicken_H
		Medium	Agri_Chicken_M
		Low	Agri_Chicken_L
Pasture	Pig	High	Past_Pig_H
		Medium	Past_Pig_M
		Low	Past_Pig_L
	Cattle	High	Past_Cattle_H
		Medium	Past_Cattle_M
		Low	Past_Cattle_L

- **Identification of crop species present**

Data on crop species were obtained from the annual crop database of the Spanish Ministry of Agriculture, Fisheries and Food (General Subdirectorate for Analysis, Coordination and Statistics), which comprises data from all Spanish provinces across three databases covering general land use, herbaceous crops or woody crops. For each province, crops statistics are recorded in hectares at the municipality level. For the present study, data from 2015 were extracted from all but eight provinces, for which data were unavailable from that year. For these eight cases, municipality values were inferred based on the most recent available municipality values as well as provincial values from 2015.

In this way, we generated a “crop database” integrating general land use, herbaceous crops and woody crops in all municipalities of Spain. This database contained 166 crops from 39 families and altogether at least 150 different taxa.

After overlaying the geographic information available, each municipality of the crop database was assigned to 1-5 of the 15 subscenarios (see Table 1). Within each scenario and animal species, a given municipality could belong to only a single manure load category, so it was assigned to the category showing the largest area in the municipality. Then, for the nine agriculture subscenarios, we calculated the mean percentage of total crop area covered by each crop and plant family, as well as the standard deviation and coefficient of variation in that percentage. Differences in crop percentages among the nine agriculture subscenarios were explored using linear mixed-effects (LME) models [66]. Crop types were considered as random factors in order to control for differences due to intrinsic characteristics of each crop category. Differences found to be significant with 95% confidence were further assessed using Tukey's post-hoc test in the emmeans package in R [67].

- **Identification of wild species present**

Data on wild plant population occurrences were obtained from the Global Biodiversity Information Facility (GBIF) database [68]. To be included, occurrence records had to have been deposited after 1980 and had to contain geographic coordinates to a precision of at least two decimal places in both latitude and longitude. Only non-crop species listed in Annex 2 of the OECD 208 method were considered as targeted species in this study. The 52 original taxa in the OECD method became 51 after species names were harmonised using the Germplasm Resources Information Network Taxonomy [69].

Georeferencing data on wild plant populations were screened for intraspecific spatial duplicates, defined as species occurrences less than 1 km apart [70, 71]. Only one of such duplicate occurrences was retained in the dataset. The cleaned set of population occurrences included 69,883 records and was intersected with every map of the agriculture scenario (pigs, cattle and chickens) and pasture scenario (pigs and cattle) in order to derive the number of species, Simpson diversity [72], and Pielou's evenness [73]. For each subscenario within the agriculture and pasture scenarios, we calculated percentages of occurrence of each wild targeted species with respect to the total occurrence of all the targeted species in that subscenario. Differences in richness, diversity and evenness across subscenarios were explored using LME models [66]. Wild species were considered as random factors in order to control for differences in the incidence values due to intrinsic features of each species. When the effects were significant with 95% confidence, a post-hoc Tukey HSD test was performed using the emmeans package in R [67].

Finally, agriculture and pasture scenarios were overlapped with a geographical information system layer that included both the Spanish Sites of Community Importance network (belonging to the Natura 2000 network; Sundseth, 2008) and the Biosphere Reserves in Spain [75]. The objective was to estimate the area of these protected networks that was subjected to some type of livestock pressure and, therefore, exposed to antibiotics.

3. Results

- **Mapping of agriculture and pasture scenarios**

Our analysis indicated that forest and shrublands covered 38.89% of the total surface in Spain; arable crops, 36.69%; and permanent grasslands, 16.71% (Table 2). In the agriculture scenario, 65.10% of the total land was arable, comprising crops (53.43%) and fallow lands (11.67%), while 11.25% of the total land was natural grassland. In the pasture scenario, 21.43% of the total land was arable (16.73% crops and 4.70% fallow lands), while 21.83% was natural grassland. An estimated 63.1-78.1% of total agricultural land area and 22.4-97.5% of total pasture land area could receive manure from livestock farms (Table A3).

Table 2
Main land cover statistics in agriculture and pasture scenarios in Spain

	Agriculture scenario			Pasture scenario			Entire Spain
	%* Mean	Std. dev.	Coeff. var.	%* Mean	Std. dev.	Coe. var.	%**
Arable land	65.10	1.95	0.03	21.43	5.84	0.27	36.69
Crops	53.43	2.29	0.04	16.73	5.11	0.31	30.17
Herbaceous crops	37.89	3.63	1.06	8.38	2.86	1.56	16.98
Woody crops	15.55	3.95	0.25	8.35	4.05	0.49	13.18
Fallow lands	11.67	0.93	0.08	4.70	1.45	0.31	6.52
Natural grasslands	11.25	1.22	0.11	21.83	4.03	0.18	16.71
Mediterranean grasslands	5.38	1.36	0.25	16.78	5.51	0.33	9.60
Natural meadows	1.23	1.18	0.95	2.54	2.54	1.00	2.17
Grazed uncultivated old agricultural land	4.64	0.63	0.14	2.52	2.72	1.08	4.95
Forests and shrublands	15.69	1.94	0.12	50.39	2.64	0.05	38.89
Neither natural nor agriculture (rest of the area)	7.96	0.81	0.10	6.34	0.54	0.09	7.71
* Calculated with respect to the total area of the municipalities belonging to each scenario							
** Calculated with respect to the total area of Spain							

The pasture scenario showed the highest spatial coverage for cattle and the lowest coverage for pigs, and the two species were also associated with, respectively, the highest and lowest percentages of surface under high manure load (Table A3). These subscenarios concentrated in the western part of the country (Figures 1b and 1d).

In contrast, the agriculture scenario extended over flat areas throughout the country (Figures 1a, 1c and 1e). The land surface under high manure load was twice as large for chickens as for cattle and pigs (Table A3).

- **Identification of crop species**

Mean percentages of total crop area covered by each crop family did not differ significantly across the three livestock species ($F = 0.58$; $p = 0.56$) or three manure loads ($F = 0.006$; $p = 0.93$). Therefore, the mean percentages for each crop family, as well as the associated standard deviations and coefficients of variation, were averaged across all agricultural subscenarios (Table 3). Herbaceous families covered 71.05% of crop land, with Poaceae the most abundant family (52.60%), while woody families covered 28.94%.

Table 3

Minimum number of crops, minimum number of taxa and mean, standard deviation and coefficient of variation of the area covered by different crop families as a percentage of the total agricultural area in the agriculture scenario.

Family	Crop Type	Min. no. of crops	Min. no. of taxa	Crop Area (% total area)		
				Mean	Std. dev.	Coeff. var.
Poaceae	Herbaceous (except cane)	21	19	52.60	5.85	0.11
Fabaceae	Herbaceous (except carob)	28	19	8.02	1.53	0.19
Convolvulaceae	Herbaceous	1	1	0.01	0.00	0.84
Cyperaceae	Herbaceous	1	1	0.00	0.00	1.56
Chenopodiaceae	Herbaceous	5	5	0.40	0.13	0.33
Malvaceae	Herbaceous	1	1	0.19	0.29	1.55
Linaceae	Herbaceous	2	1	0.27	0.36	1.34
Cannabaceae	Herbaceous	3	2	0.00	0.00	1.15
Asteraceae	Herbaceous	7	7	6.14	2.71	0.44
Brassicaceae	Herbaceous	7	6	0.84	0.36	0.43
Solanaceae	Herbaceous	7	6	0.76	0.19	0.25
Apiaceae	Herbaceous	4	3	0.08	0.02	0.30
Iridaceae	Herbaceous	1	1	0.00	0.00	0.43
Lamiaceae	Herbaceous	3	3	0.01	0.01	0.42
Caryophyllaceae	Herbaceous	1	1	0.00	0.00	0.51
Cucurbitaceae	Herbaceous	7	5	0.26	0.10	0.38
Asparagaceae	Herbaceous	1	1	0.08	0.03	0.37
Amaryllidaceae	Herbaceous	4	4	0.32	0.11	0.35
Agaricales	Herbaceous	1	1	0.01	0.01	1.17
Sown meadows	Herbaceous	1	2	0.74	0.81	1.10
Groups of various herbaceous ssp./families	Herbaceous	13	13	0.32	0.07	0.21
Rutaceae	Woody	6	6	1.86	0.83	0.44

Family	Crop Type	Min. no. of crops	Min. no. of taxa	Crop Area (% total area)		
				Mean	Std. dev.	Coeff. var.
Rosaceae	Woody (except strawberry and rose)	15	15	6.32	4.41	0.70
Moraceae	Woody	1	1	0.06	0.03	0.47
Annonaceae	Woody	1	1	0.00	0.01	1.66
Punicaceae	Woody	1	1	0.02	0.02	0.66
Lauraceae	Woody	1	1	0.04	0.06	1.36
Musaceae	Woody	1	1	0.01	0.01	1.55
Arecaceae	Woody	1	1	0.00	0.01	2.37
Cactaceae	Woody	1	1	0.00	0.00	0.53
Juglandaceae	Woody	1	1	0.06	0.04	0.66
Betulaceae	Woody	1	1	0.09	0.11	1.22
Vitaceae	Woody	1	1	5.78	2.34	0.41
Oleaceae	Woody	1	1	14.26	5.79	0.41
Capparaceae	Woody	1	1	0.00	0.00	1.34
Rubiaceae	Woody	1	1	0.00	0.00	3.00
Salicaceae	Woody	1	1	0.00	0.00	1.45
Anacardiaceae	Woody	1	1	0.00	0.00	0.00
Groups of various woody ssp/families	Woody	11	12	0.44	0.12	0.28
TOTAL		166	150	100		

Most herbaceous families in the agriculture scenario matched those listed in the OECD 208 method (Table A4). Monocotyledonae were present in 74.43% of total area in the agriculture scenario, and dicotyledonae in the remaining 23.66%. Only around 2% of crop land was covered by families not listed in the OECD 208 method. Thirteen species covered more than 1% of crop land in the agriculture scenario; the most abundant individual species were barley (31.24%) and wheat (24.96%) (Table 4).

Table 4

Ranking of herbaceous crops based on the percentage of total herbaceous crop area covered by each crop in the agriculture scenario and in Spain.*

Family	Crop	Agriculture scenario				Spain		
		Position	% Mean	Std. dev.	Coeff. var.	Position	Area (Ha)	Area (%)
Poaceae	Barley (<i>Hordeum vulgare</i> L.)	1	31.24	3.33	0.11	1	2.609.699	28.28
Poaceae	Wheat (<i>Triticum</i> sp.)	2	24.96	2.65	0.11	2	2.179.058	23.61
Asteraceae	Sunflower (<i>Helianthus annuus</i> L.)	3	8.32	3.81	0.46	3	742258	8.04
Poaceae	Corn (<i>Zea mays</i> L.; non-forage corn)	4	5.44	3.08	0.57	5	374525	4.06
Poaceae	Oat (<i>Avena sativa</i> L.)	5	4.19	0.79	0.19	4	484454	5.25
Fabaceae	Alfalfa (<i>Medicago sativa</i> L.)	6	3.72	2.17	0.58	7	257059	2.79
Poaceae	Triticosecale (<i>Triticale</i>)	7	1.84	0.42	0.23	9	176589	1.91
Fabaceae	Dried pie (<i>Pisum sativum</i> L.)	8	1.68	0.43	0.26	10	162261	1.76
Poaceae	Rye (<i>Secale cereale</i> L.)	9	1.52	0.45	0.29	11	146946	1.59
Fabaceae	Vetch (<i>Vicia sativa</i> L.)	10	1.48	0.51	0.35	15	104400	1.13

* Only crops with percentages above 1% are shown

Family	Crop	Agriculture scenario				Spain		
		Position	% Mean	Std. dev.	Coeff. var.	Position	Area (Ha)	Area (%)
Poaceae	Winter cereals for fodder	11	1.45	0.35	0.24	8	184800	2.00
Fabaceae	Forage vetch (<i>Vicia sativa</i> L.)	12	1.32	0.60	0.45	12	120187	1.30
Poaceae	Rice (<i>Oryza sativa</i> L.)	13	1.07	0.80	0.75	13	109302	1.18
Various	Sown meadows	14	1.02	1.03	1.01	6	284360	3.08
	% Total of crops above 1%		89.24					85.98
* Only crops with percentages above 1% are shown								

- **Identification of wild plant species**

Forty-six of the 51 taxa listed in Annex 3 of the OECD 208 method occur in Spain; no occurrence records with coordinates were obtained from the GBIF for *Elymus canadensis* L., *Hordeum pusillum* Nutt., *Ipomoea hederacea* Jacq., *Senna obtusifolia* (L.) H. S. Irwin & Barneby, or *Sesbania exaltata* (Raf.) Rydb. Of the 46 taxa present in Spain, 42 occurred within all five combinations of scenarios and animal species. All the families containing the 42 plant species occurred in all five combinations of scenarios and animal species.

The 15 subscenarios showed similar values for richness, Simpson diversity index and Pielou index, with the exception of pig-rearing areas under medium or high manure load in the pasture scenario, which showed lower values for these parameters (Table A5). Nevertheless, no significant differences were found in richness, diversity or evenness across livestock species (Richness: $F = 2.3$; $p = 0.15$; Simpson: $F = 2.06$; $p = 0.17$; Pielou: $F = 1.8$; $p = 0.2$) or manure loads (Richness: $F = 0.61$; $p = 0.56$; Simpson: $F = 0.3$; $p = 0.75$; Pielou: $F = 0.22$; $p = 0.8$). Therefore, results were analysed further only at the level of the two scenarios.

In the agriculture scenario, Poaceae, Asteraceae and Polygonaceae were the families with the largest percentages of occurrence, and the most abundant species in these families were *Echinochloa crus-galli* (L.) P. Beauv., *Xanthium spinosum* L. and *Rumex crispus* L., respectively (Table 5). In the pasture scenario, Primulaceae, Poaceae and Rubiaceae were the families with the largest percentages of occurrence, with *Bromus tectorum* L. and *Galium aparine* L. the most abundant species. Even if the same family occupied the largest percentage of crop area in both the agriculture and pasture scenarios, the most abundant

species from that family could differ. For example, the Poaceae species occupying the largest percentages were *Echinochloa crus-galli* and *Digitaria sanguinalis* (L.) Scop. in the agriculture scenario, but *Bromus tectorum* and *Agrostis capillaris* L. in the pasture scenario. In some cases, the same species from the same family dominated both the agriculture and pasture scenarios, as occurred with the Polygonaceae species *Rumex crispus* and *Persicaria maculosa* Gray (Table 5).

Table 5

Mean, standard deviation and coefficient of variation of the percentage of presence of wild targeted species in agriculture and pasture scenarios

Family	Species	Agriculture scenario		Pasture scenario	
		Mean±Std. dev.	Coeff. var.	Mean±Std. dev.	Coeff. var.
Apiaceae	TOTAL	0.44±0.11	0.26	0.54±0.40	0.75
	<i>Torilis japonica</i> (Houtt.) DC.	0.44±0.11	0.26	0.54±0.40	0.75
Asteraceae	TOTAL	11.25±1.19	0.11	9.35±2.43	0.26
	<i>Bellis perennis</i> L.	2.59±0.19	0.07	4.05±0.80	0.20
	<i>Centaurea cyanus</i> L.	1.73±0.43	0.25	0.83±0.53	0.64
	<i>Centaurea nigra</i> L.	1.01±0.55	0.55	1.49±1.44	0.97
	<i>Inula helenium</i> L.	0.14±0.06	0.45	0.08±0.09	1.12
	<i>Leontodon hispidus</i> L.	0.80±0.18	0.23	1.08±0.91	0.85
	<i>Rudbeckia hirta</i> L.*			0.00±0.00	1.60
	<i>Solidago canadensis</i> L.	0.12±0.12	0.94	0.06±0.06	1.13
	<i>Xanthium spinosum</i> L.	3.41±0.77	0.23	1.26±0.61	0.48
	<i>Xanthium strumarium</i> L.	1.45±0.38	0.27	0.50±0.20	0.40
	Brassicaceae	TOTAL	0.74±0.37	0.50	1.06±1.01
<i>Cardamine pratensis</i> L.		0.74±0.37	0.50	1.06±1.01	0.95
Caryophyllaceae	TOTAL	0.44±0.28	0.64	0.58±0.42	0.72
	<i>Silene flos-cuculi</i> (L.) Greuter & Bourdet	0.44±0.28	0.64	0.58±0.42	0.72
Chenopodiaceae	TOTAL	6.19±0.66	0.11	2.65±1.28	0.48
	<i>Chenopodium album</i> L.	6.19±0.66	0.11	2.65±1.28	0.48
Cyperaceae	TOTAL	1.59±0.53	0.33	0.47±0.33	0.71
	<i>Cyperus rotundus</i> L.	1.59±0.53	0.33	0.47±0.33	0.71
Fabaceae	TOTAL	9.06±0.73	0.08	8.64±4.38	0.51
	<i>Lotus corniculatus</i> L.	4.32±0.43	0.10	4.00±2.69	0.67

* Information about five populations was obtained from the Global Biodiversity Information Facility (GBIF) database [68]: one in the Past_Cattle_H subscenario, one in the Past_Cattle_M subscenario and three in the agricultural and pasture scenarios

		Agriculture scenario		Pasture scenario	
	<i>Trifolium pratense</i> L.	4.75±0.33	0.07	4.64±1.72	0.37
Hypericaceae	TOTAL	7.08±1.38	0.20	5.81±1.94	0.33
	<i>Hypericum perforatum</i> L.	7.08±1.38	0.20	5.81±1.94	0.33
Lamiaceae	TOTAL	5.67±0.91	0.16	6.97±3.25	0.47
	<i>Betonica officinalis</i> L.	1.98±0.54	0.27	2.71±1.60	0.59
	<i>Leonurus cardiaca</i> L.	0.02±0.02	0.76	0.02±0.02	1.13
	<i>Mentha spicata</i> L.	0.48±0.21	0.43	0.39±0.08	0.22
	<i>Nepeta cataria</i> L.	0.49±0.15	0.31	0.31±0.13	0.43
	<i>Prunella vulgaris</i> L.	2.70±0.35	0.13	3.54±1.70	0.48
Malvaceae	TOTAL	0.82±0.59	0.71	0.15±0.10	0.64
	<i>Abutilon theophrasti</i> Medik.	0.7±0.53	0.74	0.15±0.09	0.62
	<i>Sida spinosa</i> L.	0.10±0.10	1.04	0.00±0.01	1.62
Papaveraceae	TOTAL	8.29±1.16	0.14	6.47±2.76	0.43
	<i>Papaver rhoeas</i> L.	8.29±1.16	0.14	6.47±2.76	0.43
Plantaginaceae	TOTAL	5.84±1.34	0.23	7.23±1.78	0.25
	<i>Digitalis purpurea</i> L.	3.14±1.33	0.42	5.55±1.84	0.33
	<i>Veronica persica</i> Poir.	2.70±0.64	0.24	1.67±0.60	0.36
Poaceae	TOTAL	12.59±1.38	0.11	12.84±2.58	0.20
	<i>Agrostis capillaris</i> L.	1.59±0.49	0.31	2.46±2.05	0.83
	<i>Alopecurus myosuroides</i> Huds.	1.28±0.21	0.17	0.39±0.27	0.71
	<i>Avena fatua</i> L.	0.87±0.23	0.27	0.59±0.21	0.36
	<i>Bromus tectorum</i> L.	1.59±0.34	0.21	3.83±2.40	0.63
	<i>Cynosurus cristatus</i> L.	1.23±0.67	0.54	1.94±1.23	0.63
	<i>Digitaria sanguinalis</i> (L.) Scop.	2.30±0.36	0.16	1.27±0.28	0.22
	<i>Echinochloa crus-galli</i> (L.) P. Beauv.	2.80±0.63	0.22	1.13±0.30	0.27

* Information about five populations was obtained from the Global Biodiversity Information Facility (GBIF) database [68]: one in the Past_Cattle_H subscenario, one in the Past_Cattle_M subscenario and three in the agricultural and pasture scenarios

		Agriculture scenario		Pasture scenario	
	<i>Festuca pratensis</i> Huds.	0.27±0.19	0.71	0.35±0.36	1.02
	<i>Phleum pratense</i> L.	0.96±0.28	0.29	0.89±0.68	0.77
Polygonaceae	TOTAL	11.00±1.75	0.16	8.03±1.07	0.13
	<i>Fallopia convulvulus</i> (L.) Á. Löve	1.54±0.41	0.27	0.87±0.51	0.59
	<i>Persicaria lapathifolia</i> (L.) Delarbre	2.16±0.30	0.14	1.30±0.40	0.31
	<i>Persicaria maculosa</i> Gray	2.70±0.49	0.18	1.60±0.35	0.22
	<i>Persicaria pensylvanica</i> (L.) M. Gómez	0.01±0.01	1.11	0.00±0.01	2.45
	<i>Rumex crispus</i> L.	4.59±0.64	0.14	4.26±1.26	0.30
Primulaceae	TOTAL	8.55±1.88	0.22	16.48±11.77	0.71
	<i>Lysimachia arvensis</i> (L.) U. Manns & Anderb.	8.55±1.88	0.22	16.48±11.77	0.71
Ranunculaceae	TOTAL	0.99±0.28	0.28	1.26±0.99	0.78
	<i>Ranunculus acris</i> L.	0.99±0.28	0.28	1.26±0.99	0.78
Rosaceae	TOTAL	1.41±0.45	0.32	2.02±1.41	0.70
	<i>Geum urbanum</i> L.	1.41±0.45	0.32	2.02±1.41	0.70
Rubiaceae	TOTAL	7.73±1.42	0.18	9.44±2.71	0.29
	<i>Galium aparine</i> L.	6.83±1.39	0.20	8.25±3.32	0.40
	<i>Galium mollugo</i> L.	0.90±0.29	0.33	1.19±0.76	0.64
* Information about five populations was obtained from the Global Biodiversity Information Facility (GBIF) database [68]: one in the Past_Cattle_H subscenario, one in the Past_Cattle_M subscenario and three in the agricultural and pasture scenarios					

The coefficients of variation in the percentages of occurrence of the targeted wild species were low and ranged from 0.20 to 2.45 at the species level, and from 0.13 to 0.95 at the family level (Table 5).

We detected livestock pressure on 25% of the lands protected within the Spanish Sites of Community Importance or the Biosphere Reserves (Figure 2). In fact, 75% of these protected areas was estimated to be exposed to antibiotics in the pasture scenario (Figure 2). The subscenarios predicting the largest areas of exposure risk in protected lands were the cattle-rearing lands under low or medium manure load in the pasture scenario (Table A6). The subscenarios predicting the smallest areas of exposure risk were pig-rearing lands under high manure load in the agriculture and pasture scenarios.

4. Discussion

- **Agriculture and pasture scenarios and subscenarios**

Our maps for agriculture and pasture subscenarios (Figure 1) reveal a heterogeneous “patchwork” reflecting the diverse influences on agricultural and livestock activities in Spain, which include bioclimatic, socioeconomic and policy factors [46, 76, 77].

Geographical areas under high manure load in our study correspond to high productive regions of arable land, pasture and meadows [78]. In the agriculture scenario, areas under high manure load were located predominantly in the Iberian central high plateau and the Ebro River Basin, which feature mainly arable lands with herbaceous crops, especially cereals (Figures 1a, 1c and 1e). We also observed areas under high manure load in southern Spain and along the Mediterranean coast that were associated with rearing of chickens (Figure 1e). The wider distribution of chickens than of cattle or pigs may reflect the stronger economic constraint on chicken farms to be closer to markets, rather than the influence of climatic or other environmental factors [79].

In the cattle pasture scenario, geographical areas under high manure load were present in the humid northern provinces, especially along the north and central mountain ranges, which are characterised by rich pastures and meadows. Such areas lay also in the west and southwest of the Peninsula, where pasture lands known as “dehesas” are abundant [51]. Dehesas, an agroforestry system that harmoniously combines pastures and trees, are one of the most ecologically valuable and economically important rural landscapes on the Iberian Peninsula [78]. The distribution of dehesas coincides exactly with that of pig-rearing pastures in our study (Figure 1b), even though cattle also graze on dehesas [80].

The agriculture scenario showed extensive surface devoted to arable land, mainly for herbaceous crops such as barley and wheat, and to a lesser extent for woody crops, mainly olive, vineyards and fruit trees. In contrast, more than two thirds of the land surface in the pasture scenario was woody vegetation (forest and shrublands) and natural grass. Natural grasslands, which include meadows, rough grassland and rough grazing rangeland, are dominated by herbaceous species and are the main food source for extensively managed livestock in Spain [51]. Shrublands and open forests also play an essential role in livestock farming when green grass is in short supply [51].

- **Monocotyledonous crop species in the agriculture scenario**

Plant toxicity assays are designed to be rapid, simple and reproducible, which helps explain why the OECD 208 method includes only herbaceous, not woody, species as sentinels for assessing risk of environmental effects due to antibiotics given to livestock. Indeed, herbaceous crop plants are the focus of efforts to fertilise arable lands with manure. In England and Wales, manure is applied to arable crops of cereals (wheat, barley and maize), sugar beet and potatoes, as well as to land for grazing and silage [59, 60]. In France, 36.5% of total manure used for fertiliser is spread onto grassland, 39.6% onto land for maize, 12.9% onto land for cereals and 7.9% onto land for oilseed or protein crops [58].

We found that in Spain, the Monocotyledonae group and concretely the Poaceae family dominated herbaceous crop species in the agriculture scenario (Table 4). This reflects that cereals cover more land in Spain than any other crop, occupying 71% of the herbaceous crop area. This broad distribution reflects, in part, the plants' adaptability to different soils and climatic conditions. At the national level, cereals account for 7.1% of agrarian production and 12.3% of vegetable production [81]. We found that barley, wheat and corn occupied the top positions in the agriculture scenario (Table 4), consistent with their status as the main cereals produced in Spain [81]. The situation is likely to be similar at the level of the entire EU, which is the largest wheat and barley producer worldwide and which accounts for 6% of global corn production [81]. Wheat, barley and corn are included in Annex 2 of the OECD method.

The cereals oat, triticale, rye and rice are also cultivated in Spain and all were identified in the agriculture scenario, but each of them accounted for less than 5% of the herbaceous crop area. A similar pattern occurs at the EU level, where rye accounts for 2.0% and rice for 0.4% of the total arable land [82]. All these cereal species except triticale are listed in Annex 2 of the OECD 208 method. Triticale is cultivated for winter cereal-legume forage intercrops, but legumes are necessarily more abundant in such intercrops in order to maintain feeding values [83].

The Liliaceae family was found to occupy 0.5% of the herbaceous crop area in the agriculture scenario, with the most abundant crops being onion (0.25%) and garlic (0.21%). Onion is the only species of this family listed in Annex 2 of the OECD 208 method, and it is the second most cultivated fresh vegetable in the EU. However, animal manure is not suitable for fertilising onion or other horticultural crops because bacteria, parasites and viruses in manure can contaminate the food supply [84] and lead to bioaccumulation of contaminants such as veterinary antibiotics in stems and roots [85, 86]. Nevertheless, manure can be composted for a long period and the resulting fertiliser can be considered safe for use on vegetable crops [87].

- **Dicotyledonous crop species in the agriculture scenario**

Dicotyledonae herbaceous crop species covered approximately 25% of the agriculture scenario (Table A4). The families Fabaceae and Asteraceae clearly predominated (19%), occupying the top four positions by land cover: an Asteraceae species (sunflower), followed by three Fabaceae species of alfalfa, vetch, and dried pie (Table 4). All these species except alfalfa are included in Annex 2 of the OECD 208 method.

Sunflower is an oilseed species, so it is used for human and animal consumption, fuel and industrial purposes, giving it high economic value [88]. Oilseed species come from different Dicotyledonae families: rape, which accounts for 59% of total oilseed production in the EU, comes from the Brassicaceae family; sunflower, from Asteraceae; and soybean, from Fabaceae (EC, 2021). Of these oilseed species, only sunflower and rape have a notable presence in Spain [89]; rape occupied 0.92% of the agriculture scenario, placing it at 15th position in terms of land cover.

Alfalfa and vetch are fodder crops, although vetch is also used for human consumption. Fodder crops comprise legumes, cereals, sown meadows and other crops (Eurostat, 2020b). Fodder crops are

important for extensively managed livestock in Spain [51]. Among the four most abundant fodder species in our analysis, alfalfa is by far the most important in Spain (Table 4) and the EU [51]. Vetch species are also frequently used in combination with a cereal such as oat or barley or with a pasture grass such as Italian ryegrass [51, 83].

Dried pie is a protein-rich crop, and such crops are a major source of amino acids for human and animal nutrition. Dried pie ranked eighth for land cover in the agriculture scenario (Table 7), and it is the most widespread protein-rich crop in Spain [89]. At the EU level, pea, bean and lupine are major cultivated protein crops [90].

Among the other dicot families and crop species listed in Annex 2 of the OECD 208 method, only the Chenopodiaceae family and sugar beet species have some importance in Spanish agriculture, accounting for 0.5% of herbaceous crops (20th position). The EU is the world's leading producer of sugar beet, accounting for around 50% of global production [90]. Most sugar beet in the EU is grown in northern Europe, where the climate is more suitable for this crop [91].

- **Sentinel crop species in the agriculture scenario**

The European Medicines Agency recommends that assessments of risk from veterinary antibiotics include plant species that are grown on agricultural land that are fertilised with manure [92]. It would also be advisable to take into account the intrinsic susceptibility of plant species to antibiotics [37], but such data are nearly absent from the literature [93]. Thus, given the currently available data, the best species for risk assessments may be those that are abundant on agricultural land and that receive manure fertiliser.

Given that Poaceae is the crop family with the largest area in the agriculture scenario, it would be advisable to consider at least two monocot species from among barley, wheat or corn in the risk assessment of antibiotics used to treat livestock species. Such assessment should also consider the dicot species of sunflower (Asteraceae) as oilseed, dried pie (Fabaceae) as a protein-rich crop, as well as alfalfa and vetch (Fabaceae) as fodder crops. It may also be helpful to consider the oilseed rape (Brassicaceae) and sugar beet (Chenopodiaceae), since both are widely cultivated in the EU.

- **Wild species in agriculture and pasture scenarios**

The Mediterranean Basin is one of the 25 biodiversity hotspots around the globe where many endemic species are losing their habitat [94]. The only hotspots of importance in Europe are the Mediterranean Basin and the Caucasus, both of which are extremely vulnerable to climate change [95]. As a representative region of the Basin, Spain has among the greatest diversity of ecosystems, habitats and natural species in the EU, housing more than half of the Union's species of vertebrates and vascular plants as well as 65% of its priority habitats [4].

Most pasture areas serve for grazing by livestock and/or wild ungulates, but they also provide other valuable regulation and cultural ecosystem services [78]. Spanish pasture lands contain an abundance of

Poaceae species as well as numerous other families catalogued in the Habitats of Community Interest under the EU Habitats Directive (92/43/EEC) (San Miguel et al., 2016). Our results highlight *Lysimachia arvensis* (L.) U. Manns & Anderb. (Primulaceae) and *Galium aparine* (Rubiaceae) as the most frequent species (Table 5). *Lysimachia arvensis* has been reported to cause cattle death in Uruguay and potentially also sheep death in Australia [96]. In contrast, cleaver (*Galium aparine*) may support plant biodiversity [97]. The following species appear in Annex 3 of the OECD method and have been described as most abundant in Spanish pasture lands [51], which may make them useful for assessing risk from veterinary antibiotics: *Agrostis*, *Bromus* and *Festuca* within the Poaceae family, as well as *Trifolium* and *Lotus* within the *Fabaceae* family. However, in the pasture scenario, species from this genera accounted for only 2-5% of all wild species, while *Festuca* species accounted for <1%. These species may therefore not be sufficiently representative. In other words, Annex 3 does not include most of the dominant plant species on Mediterranean pasture lands.

Arable field margins provide a wide range of ecosystem services in rural landscapes and limit the negative effects of competitive and dominant species on crop productivity [98]. Plant diversity in these habitats has declined in recent decades due to agricultural intensification [97, 99–102]. Thus, one of the main challenges of agriculture worldwide is to balance crop productivity and biodiversity maintenance.

In typical Mediterranean cereal cropping systems in the Guadalquivir river basin in southern Spain, Pallavicini et al. (2020) identified 306 weed species, of which only 10 are catalogued in the OECD Annex 3. Among these catalogued species, the four most abundant were also among the five most frequent in our agriculture scenario: *Lysimachia arvensis*, *Galium aparine*, *Papaver rhoeas* L. and *Chenopodium album* L. Similarly, in cereal fields of the Ebro basin in northeastern Spain, Cirujeda et al. (2011) identified 175 weed species. Only 6 of the most abundant species (occurring in >10% of the surveyed fields) are included in the OECD Annex 3, with *Papaver rhoeas* the most abundant. This similarity in most abundant species between our mapped scenarios and previous fieldwork suggests that our analysis based on the GBIF data is reliable.

In addition, our results indicate the relevance of *Hypericum perforatum* L., the fourth most frequent wild species in Spain among those in Annex 3 (Table 5). This species was not inventoried by Pallavicini et al. (2020) or Cirujeda et al (2011), which may indicate that this moisture-loving species does not prefer the hot, dry conditions in the Ebro and Guadalquivir river basins.

Cirujeda et al. (2011, 2019) identified several extremely abundant weed species in Spanish cereal fields not included in the OECD 208 method, including *Avena sterilis*, *Convolvulus arvensis*, *Dactylis glomerata* L., *Lolium rigidum* and *Sonchus oleraceus* L. The role of weeds in supporting biological diversity within crop fields has been studied by Marshall (2003) and Storkey (2006). From a total of 32 species identified in those studies as important for biodiversity, only five are included in Annex 3 of the OECD 208 method. All of them were present in our agriculture scenario: *Chenopodium album* (Chenopodiaceae), *Galium aparine* (Rubiaceae) and *Papaver rhoeas* (Papaveraceae) accounted for 5-8% of the total occurrence

records of all targeted species in this scenario, while *Persicaria maculosa* and *Fallopia convolvulus* (Polygonaceae) accounted for 1.5-2.7% of the total occurrence records (Table 5).

- **Sentinel wild species in agriculture and pasture scenarios**

Our analysis suggests that assessments of risk from veterinary antibiotics should take into account *Papaver rhoeas*, *Galium aparine* and *Chenopodium album* as sentinel wild species. These species are important for biodiversity and they are abundant among targeted wild species in our agriculture scenario.

Our analysis of the pasture scenario suggests that risk assessments should not take into account *Lysimachia arvensis*. Even though this species belongs to the most abundant family (Primulaceae), the plant has been linked to livestock death (Roche et al., 2012). Instead, our analysis suggests that risk assessments should include *Bromus tectorum* or *Agrostis capillaris* from the Poaceae family, as well as *Trifolium pratense* or *Lotus corniculatus* from the Fabaceae family. All these species are relatively abundant among targeted wild species and they are dominant in Mediterranean grasslands [51]. The species *Galium aparine* (Rubiaceae) could also be considered, since it was the second most frequent species in our pasture scenario and it can support biodiversity.

While all the above mentioned species have been listed in Annex 3 of the OECD 208 method, there are other weed species at the margins of arable fields and on pasture lands that could be useful in assessments of risk from veterinary antibiotics but are not listed in Annex 3. These species may include those identified as the most frequent in surveys of field boundaries [99, 100, 102] and pasture lands (San Miguel et al., 2016) and that support biodiversity or food security. For example, *Sonchus oleraceus* is one of the most frequent weed species at field boundaries in Spain [99], and it is important for biodiversity [101]. Some Poaceae species, such as *Avena sterilis*, *Dactylis glomerata*, *Hordeum murinum* and *Lolium rigidum*, are also frequent and are included on the Prioritised Spanish Checklist of Crop Wild Relatives [103], which compiles alternative crop species crucial to ensure food security.

The identification of sentinel species may be especially beneficial for biodiversity conservation within the Spanish Sites of Community Importance network and Biosphere Reserves, given that up to 75% of these protected areas may be vulnerable to phytotoxic effects from veterinary antibiotics, according to our pasture scenario. Future work to consider new “sentinel” species not currently in Annex 3 will also need to evaluate how feasible it is to develop standardised testing protocols akin to the OECD 208 method. This will depend on the commercial availability of seeds and whether they meet the conditions for laboratory assays (e.g. germination rate $\geq 70\%$ and survival rate $\geq 90\%$).

5. Conclusions

Based on geographical information systems mapping, we propose the inclusion of certain sentinel crop and wild species in assessments of environmental risk from antibiotics used to treat livestock in Mediterranean Europe. By combining georeferenced layers on livestock density and land cover, we generated agriculture and pasture scenarios for Spain as a representative country in Mediterranean

Europe. Then we identified which of the crop and wild plant species in the OECD 208 method were present in the scenarios. Finally, we assessed the suitability of the species for risk assessments based on various criteria. This led us to propose barley, wheat, corn, sunflower, dried pie, alfalfa, vetch, oilseed rape and sugar beet as sentinel crop species in the agricultural scenario. As sentinel wild species, we propose *Bromus tectorum*, *Agrostis capillaris*, *Trifolium pratense*, *Lotus corniculatus* and *Galium aparine* in the pasture scenario; and *Papaver rhoeas*, *Galium aparine* and *Chenopodium album* in the agriculture scenario. We also urge the consideration of several weed species common at field boundaries or on pasture lands that are not listed in the OECD 208 method but that contribute substantially to biodiversity.

Abbreviations

GBIF: Global Biodiversity Information Facility

LME: linear mixed-effects

REGA: Spanish Register of Livestock Holdings

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Data from Spanish Register of Livestock Holdings (REGA) are not available due to data privacy laws.

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Authors' contributions

RMG: contributed in Formal analysis, Investigation, and Writing of the Original Draft

JFM contributed in Methodology, Formal analysis, Resources, Data Curation, Visualization and Writing – Original Draft

AR contributed in Methodology and Formal analysis -kernel models, geostatistical procedures and mixed effects models-. He collaborated in the Writing Review and Editing.

AT responsible of Conceptualization, provision of data and Writing, Review and Editing

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Figures

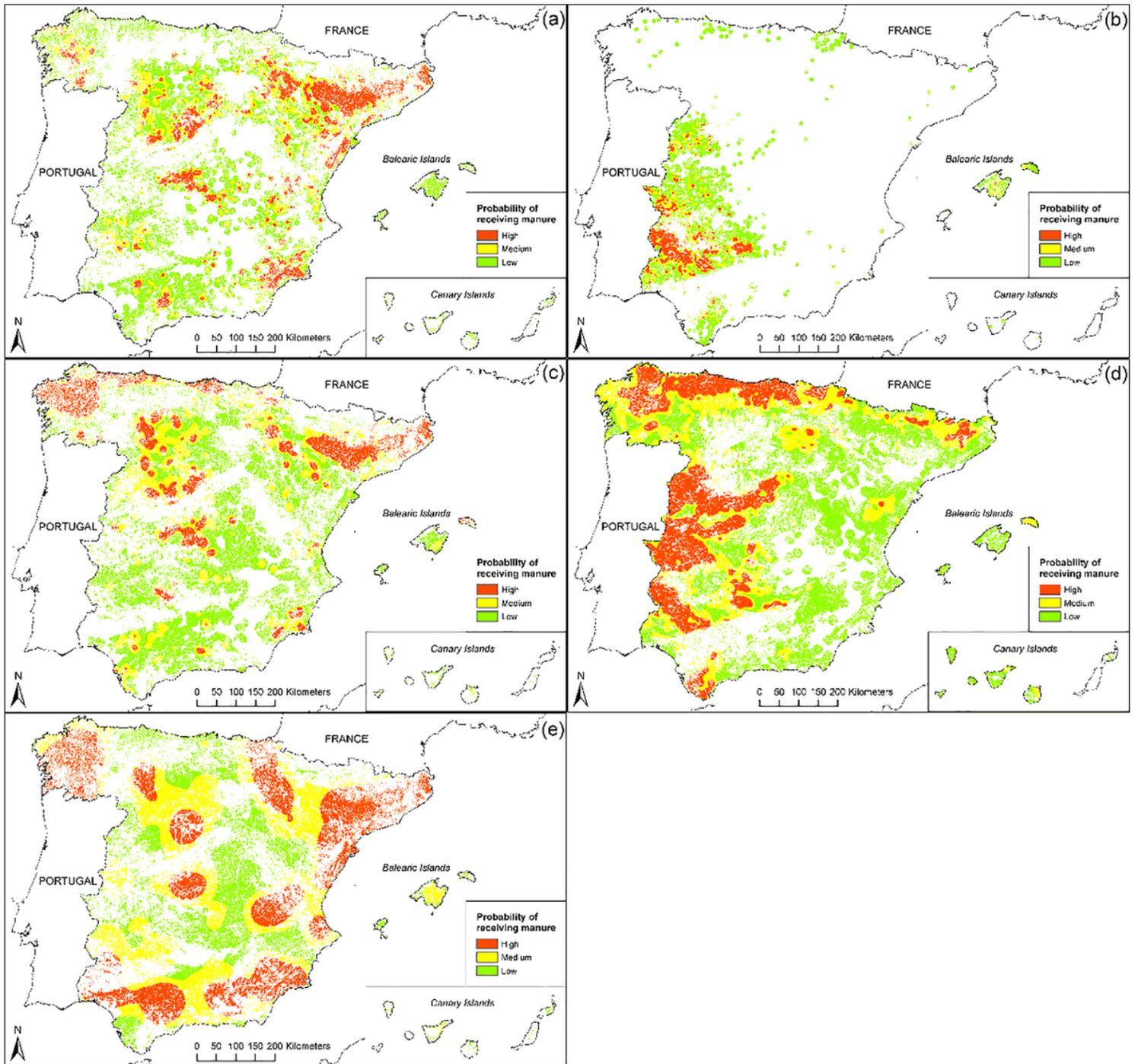


Figure 1

Probability of receiving manure from: (a) pig in agricultural scenarios; (b) pig in pasture scenarios; (c) cattle in agricultural scenarios; (d) cattle in pasture scenarios; (e) chicken in agricultural scenarios.

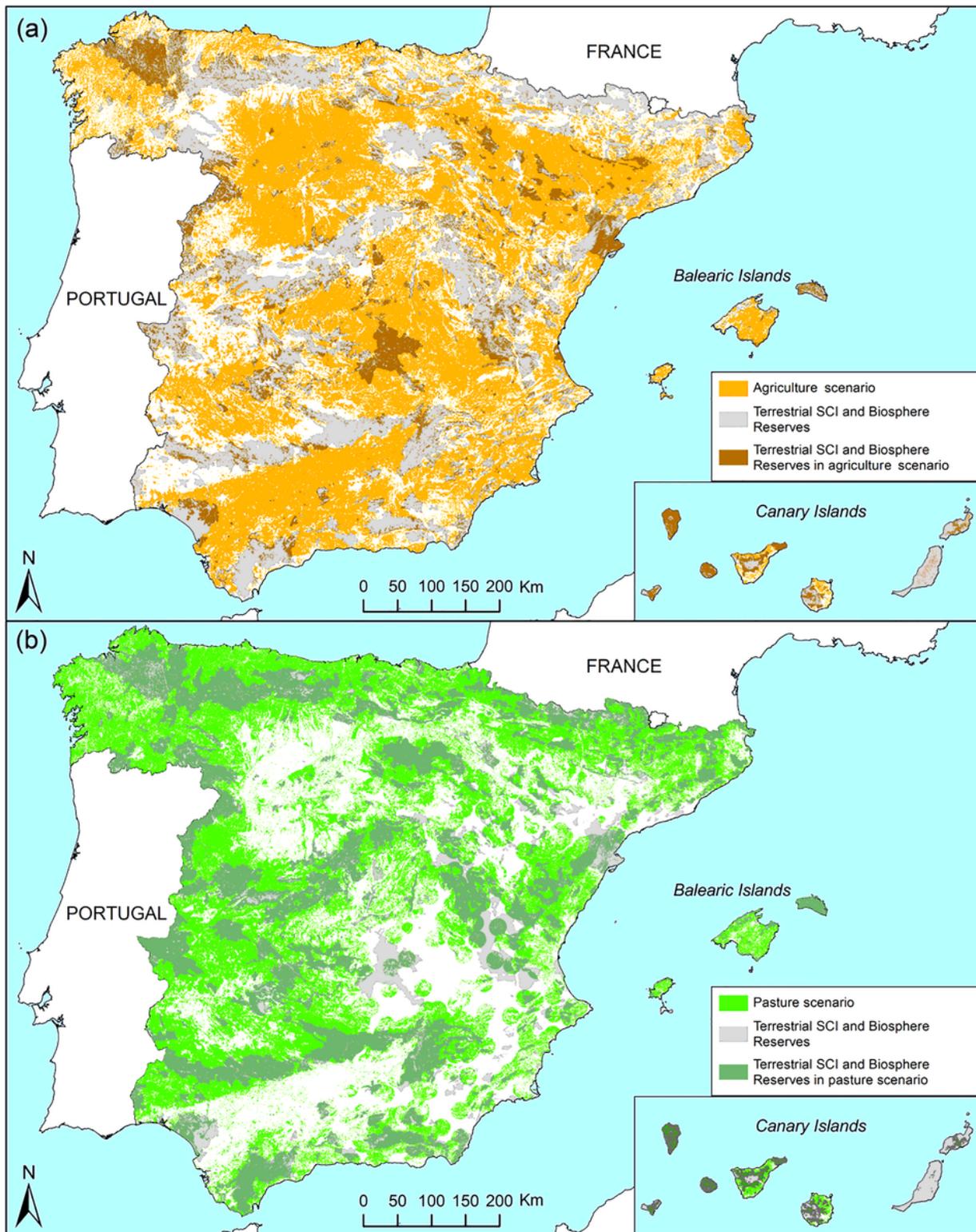


Figure 2

Overlap of the Sites of Community Importance (SCI) and Biosphere Reserves with agricultural (a) and pasture scenarios (b). Dark coloured areas correspond to areas of agricultural (orange) and pasture (green) scenarios, respectively, located inside a SCI or a Biosphere Reserve.

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